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Induction of Hepatitis C Virus-Specific Cytotoxic T Lymphocytes in Mice by an Intrahepatic Inoculation with an Expression Plasmid

Akira Kamei,*,† Shigenori Tamaki,† Hiroyuki Taniyama,‡ Shiki Takamura,* Yuki Nishimura,* Yumiko Kagawa,‡ Satori Uno-Furuta,*,† Masahiko Kaito,† Gisen Kim,* Masaaki Toda,* Yoshiharu Matsuura,§ Tatsuo Miyamura,§ Yukihiko Adachi,† and Yasuhiro Yasutomi*,[1]

*Department of Bioregulation and †Third Department of Internal Medicine, Mie University School of Medicine, Mie 514-8507, Japan; ‡Department of Veterinary Pathology, Rakunogakuen University, Hokkaido 069-0836, Japan; and §Department of Virology II, National Institute of Infectious Diseases, Tokyo 162-8640, Japan

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We assessed the possibility of intrahepatic inoculation with a plasmid encoding hepatitis C virus (HCV) proteins to elicit HCV-specific cytotoxic T lymphocytes (CTL) in mice as a conventional animal model of HCV infection. BALB/c mice were intrahepatically or intramuscularly inoculated with an expression plasmid DNA encoding HCV structural proteins under the control of the elongation factor 1-α promoter. Expressions of HCV-core protein and envelope proteins (E1 and E2) in hepatocytes were detected immunohistochemically 6 days after inoculation. CTL responses were examined using target cells either pulsed with a specific peptide or infected with a recombinant vaccinia virus expressing HCV structural protein. Both intrahepatically and intramuscularly DNA-inoculated mice developed $CD8^+$, MHC class I-restricted CTL responses that recognized the peptide pulsed as well as HCV proteins expressing target cells. These studies demonstrated the usefulness of a murine model of HCV infection induced by direct intrahepatic DNA inoculation for understanding the immunopathogenic mechanisms in HCV infection.

Key Words: CTL; DNA vaccine; animal model; HCV.

INTRODUCTION

Hepatitis C virus (HCV) is one of the major agents of chronic hepatitis and liver disease worldwide (Choo *et al.,* 1989; Houghton *et al.,* 1991; Kuo *et al.,* 1989). Infection with HCV leads to chronic hepatitis in more than 50% of cases, and some of these cases progress to cirrhosis and perhaps hepatocellular carcinoma (Saito *et al.,* 1990). While the pathogenic mechanism of progression of hepatitis caused by HCV has not yet been clearly elucidated, much information has accumulated showing that virus-specific cytotoxic T lymphocytes (CTL) play a critical role in the host cellular immune response against HCV (Imawari *et al.,* 1989; Rehermann *et al.,* 1996). CTL might contribute to hepatocellular damage in HCV infection during the course of killing virus-infected cells. Thus, identification of immunodominant epitopes that elicit antiviral CTL might contribute to an understanding of the pathogenesis of HCV infection.

An animal model for HCV infection is required both for vaccine development and for understanding the pathogenesis of HCV infection. Although the chimpanzee is the only known animal model of HCV infection, chimpanzees are difficult to acquire as laboratory animals. HCV and HLA transgenic mice have therefore been employed for vaccine development and for examination of HCV pathogenesis (Koike *et al.,* 1995; Matsuda *et al.,* 1998; Moriya *et al.,* 1995; Pasquinelli *et al.,* 1995; Shirai *et al.,* 1995). Recently, it was reported that HCV core transgenic mice that had hepatic steatosis early in life developed hepatic tumors after the age of 16 months (Moriya *et al.,* 1997). These reports suggest that the mouse system might be useful as an animal model for exploring a novel approach to understanding the pathogenesis of HCV infection. HCV transgenic mice, however, cannot be used to assess obvious immune responses against HCV antigens because of the expression of inserted HCV gene products recognized as endogenous autologous antigens. Thus, animal models showing immune responses are required for studying the pathogenesis of HCV infection. In the present study, we established a novel animal model of HCV infection by a single intrahepatic immunization with plasmid DNA encoding HCV structural proteins, and we assessed the usefulness of the induction of HCV-specific CTL for understanding the pathogenesis of HCV infection by HCV-specific immune responses.

[1] To whom correspondence and reprint requests should be addressed at Department of Bioregulation, Mie University School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan. Fax: +81-59-231-5225. E-mail: yasutomi@doc.medic.mie-u.ac.jp.

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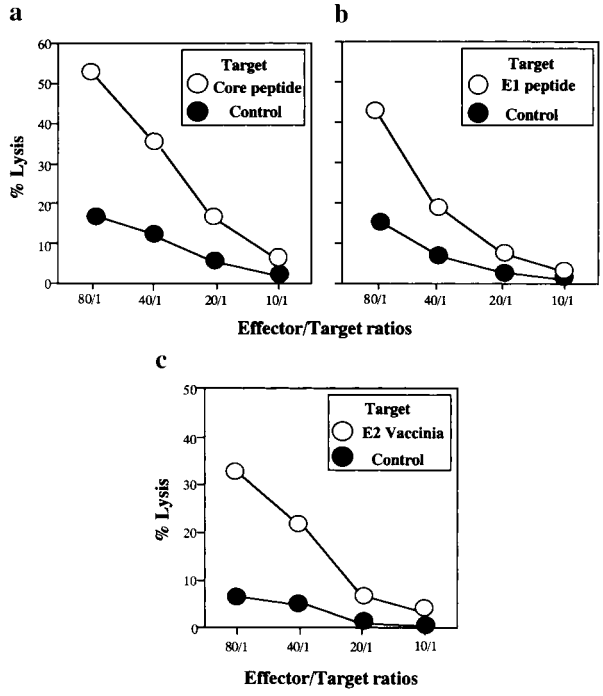

FIG. 1. Spleen cells from mice intramuscularly immunized with pEFCE1E2 develop HCV-specific lytic activities. Cytolytic activities against core (a) and E1 (b) were assessed using CTL epitope peptide-labeled (Bruna-Romero *et al.,* 1997; Saito *et al.,* 1990) target cells. Target cells infected with rVV-HCV E2 were used for measuring cytotoxic activities against E2 (c). Open symbols indicate the lysis of target cells labeled with the CTL epitope peptide (HCV core or E1) or infected with rVV-HCV E2, and closed symbols indicate the lysis of control peptide-labeled or control rVV-infected target cells. The data represent the mean percentage of the specific lysis values obtained from five mice.

RESULTS

Induction of HCV-specific cytotoxic activities by a single intramuscular immunization with plasmid DNA containing the elongation factor 1-α (EF1-α) promoter

To confirm the induction of HCV-specific effector cells by intramuscular immunization of the pEFCE1E2 plasmid, spleen cells obtained from one-shot DNA-immunized mice were assessed for their ability to lyse core, E1 peptide-pulsed, recombinant vaccinia virus (rVV)-HCV E2-infected MHC-matched target cells. The pEFCE1E2-immunized mice generated HCV-specific effector responses even after a single immunization (Fig. 1). The pEFCE1E2 immunization of mice generated effector cell responses capable of recognizing not only peptide epitopes (Figs. 1a and 1b) but also endogenously processed viral proteins (Fig. 1c). Those effector cells were $CD8^+$, MHC class I-restricted CTL (data not shown), and these results were in accordance with those obtained in our previous study (Nishimura *et al.,* 1999).

HCV structural proteins are expressed in liver cells in mice intrahepatically immunized with pEFCE1E2

To avoid the influence of injection damage, we used pEFCE1E2, which can induce immune responses with only a single immunization (Nishimura *et al.,* 1999). In fact, no pathological changes were detected in control sections that had been administered a single injection of the control plasmid. Immunohistochemical examination of the livers of mice intrahepatically immunized with pEFCE1E2 was performed 6 days to 16 weeks after inoculation with the plasmid. HCV structural proteins were observed in hepatocytes (Figs. 2a, 2b, and 2c) and other cells in mouse liver (e.g., endothelial cells; data not shown). These HCV structural proteins were observed for 16 weeks after injection, and a positive border was clearly observed only in the electroporated area. These HCV structural protein-expressing cells were observed in 2 to 7% of hepatocytes in injected liver, and there was no tendency to changes in expression rate of HCV structural proteins and or upon histological examination in the observation period. Moreover, there was no significant pathological abnormality in other organs. Based on these findings, injected plasmid DNA was inserted into the cells only in the area in which electric pulses had been administered. During the observation period, the only pathologic change detected in the livers of mice inoculated with pEFCE1E2 was mild inflammation (Fig. 2e), and no pathological changes were detected in the livers inoculated with the control plasmid (Fig. 2f).

HCV-specific effector cells are elicited in mice intrahepatically immunized with the plasmid

Spleen cells obtained from mice intrahepatically immunized with pEFCE1E2 were assessed for lytic activities against rVV-HCV core- or rVV-HCV E1-infected target cells. Mice intrahepatically immunized with pEFCE1E2 generated HCV-core- and HCV-E1-specific effector cells 2 weeks after inoculation (Figs. 3a and 3b). Moreover, these effector cells also lysed target cells labeled with HCV-core or -E1 CTL epitope peptides (Figs. 3c and 3d). However, no such effector cells were observed in the spleens of mice intrahepatically immunized with the control plasmid. Thus, intrahepatic immunization with pEFCE1E2 induces the generation of effector cells that recognize not only HCV structural proteins endogenously processed by target cells but also CTL epitope peptide-labeled target cells. HCV structural protein-specific antibody responses were not detected by immunofluorescence analyses until 6 weeks after injection (data not shown).

pEFCE1E2 intrahepatic immunization induces the generation of $CD8^+$ effector cells

HCV-core and -E1 CTL epitope peptide-specific effector cells in mice immunized with pEFCE1E2 were cul-

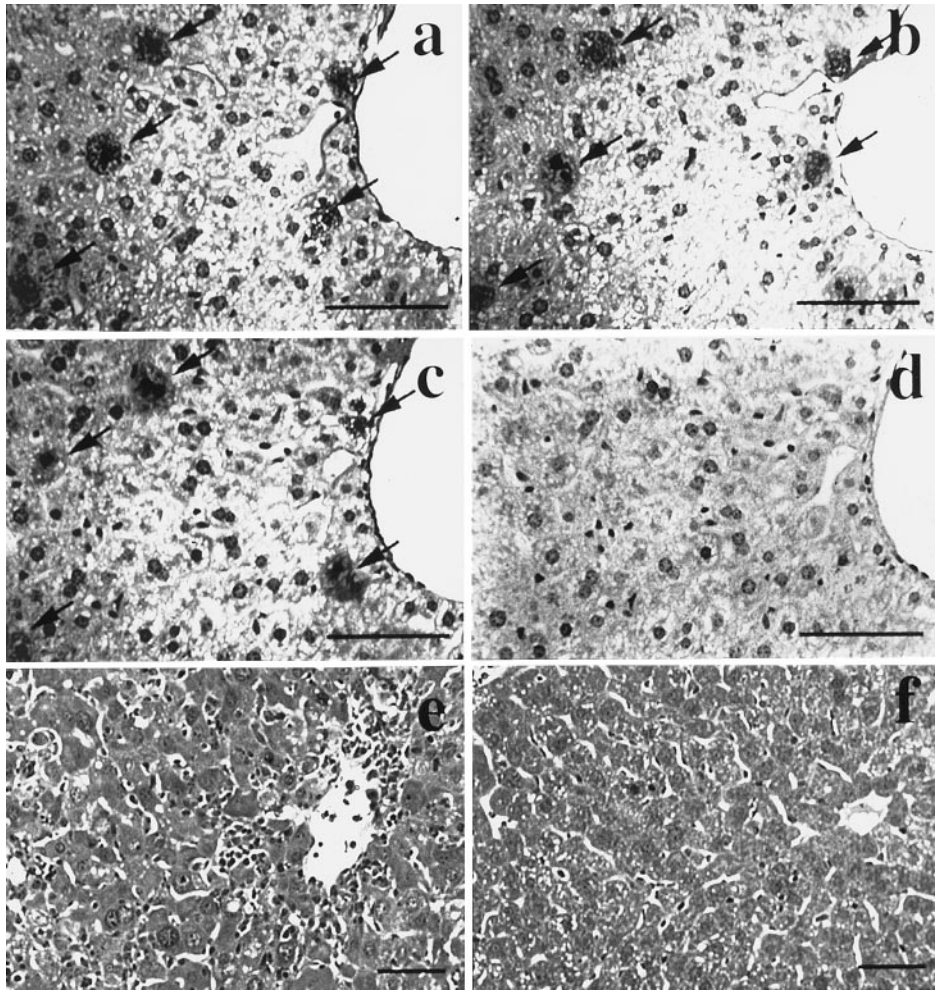

FIG. 2. Immunostaining of serial sections of liver tissue from a mouse 2 weeks after intrahepatic inoculation with pEFCE1E2. HCV structural proteins were observed in hepatocytes. The same cells expressed HCV-core (a), -E1 (b), and -E2 (c) proteins (arrows). (d) Control to immunostaining. HE staining of sections of liver tissue from mice after intrahepatic inoculation with pEFCE1E2 indicated mild inflammation (e) but not after intrahepatic inoculation with control plasmid (f). Bar represents 50 μm.

tured in medium containing anti-CD4 or anti-CD8 monoclonal antibodies (mAbs) during a ^{51}Cr release assay. Anti-CD8 mAbs inhibited cytolysis against target cells pulsed with HCV-core and -E1 peptides, whereas anti-CD4 mAbs did not affect effector cell function (Fig. 4). These results indicated that effector cells expressed CD8 and used this molecule to lyse the target cells.

Effector cells from mice intrahepatically immunized with pEFCE1E2 show MHC class I restriction

Lytic activities of HCV-core CTL epitope peptide-specific effector cells against MHC-matched or -mismatched target cells labeled with HCV-core and -E1 CTL epitope peptides were assessed. These peptide-specific effector cells lysed MHC-matched H-2^d target cells but not mismatched H-2^b target cells pulsed with CTL epitope peptides (Fig. 5a). Moreover, the functions of these HCV-core and -E1 specific effector cells were inhibited by anti-H-2D^d and H-2L^d mAbs, respectively, but not by an antibody to H-2K^d (Fig. 5b). These results indicated that intrahepatic pEFCE1E2 immunization elicited CD8$^+$ and MHC class I-restricted CTL and suggested that mice could be used as a model of HCV infection.

DISCUSSION

Studies of the pathogenesis and the prognosis of HCV infection have been hampered by the lack of small and conventional experimental animals that support replication of HCV. The chimpanzee is the only animal exhibiting susceptibility to HCV infection and has been used for studies on the replication of and the immune response against HCV (Erickson *et al.,* 1993; Farci *et al.,* 1992, 1999). Recently, full-length RNA transcripts from complete cDNA clones of HCV were shown to be infectious to chimpanzees by intrahepatic injection (Kolikhalov *et al.,* 1997). Although the chimpanzee is an ideal experi-

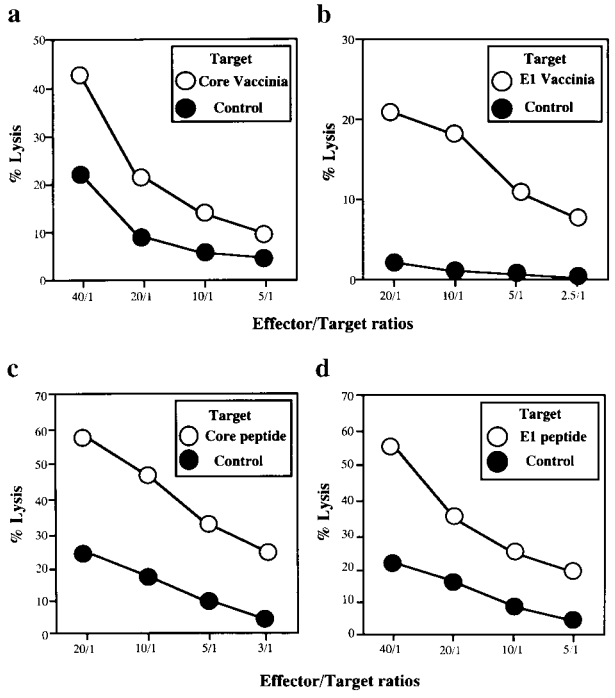

FIG. 3. Spleen cells from mice intrahepatically inoculated with plasmid DNA develop HCV-specific lytic activities. Cytolytic activities against core (a) and E1 (b) were assessed using target cells infected with rVV-HCV core and rVV-HCV E1, respectively. These effector cells also lysed target cells labeled with the CTL epitope peptide of HCV core (c) and E1 (d). Open symbols indicate the lysis of target cells infected with rVV-HCV core or rVV-HCV E1 or labeled with CTL epitope peptide (HCV core or E1), and closed symbols indicate the lysis of control peptide-labeled or control rVV-infected target cells. The data represent the mean percentage of the specific lysis values obtained from five mice.

mental model for HCV infection, chimpanzees are difficult to obtain for laboratory experiments. Thus, models using laboratory animals that are easy to obtain are needed in order to understand the effects of HCV infections. To overcome this difficulty, various types of HCV transgenic mice bearing HCV cDNA have been established for investigating the mechanisms of hepatitis and tumorigenicity induced by HCV infection (Kawamura *et al.,* 1997; Koike *et al.,* 1995; Matsuda *et al.,* 1998; Moriya *et al.,* 1995, 1997; Pasquinelli *et al.,* 1995). Transgenic mice may be a good model system for elucidating the roles of these proteins in the pathogenesis of hepatitis or hepatic tumorigenesis; however, transgenic mice do not provide a perfect system for understanding the pathogenesis of HCV infection. First, HCV gene products express not only the original target organ but also other nontarget cells. To overcome this problem, a liver-specific expression promoter has been used in some cases (Kawamura *et al.,* 1997; Matsuda *et al.,* 1998; Moriya *et al.,* 1995; Pasquinelli *et al.,* 1995). Second, it is difficult to establish transgenic mouse lines in some cases. Most transgenic mice expressing full-length HCV DNA have died after breeding, and breeding lines of female mice expressing HCV-core DNA have been difficult to maintain (Matsuda *et al.,* 1998; Moriya *et al.,* 1997). Finally, immune responses in models of HCV infection are very important for understanding the pathogenesis of HCV infection; however, immune responses against exogenous transgene products in transgenic mice are difficult to detect in most cases because exogenous gene products are probably recognized as endogenous antigens. Recently, excellent transgenic mouse lines with clear immune responses against HCV gene products have been established by using a Cre/*lox*P system (Wakita *et al.,* 1998). The HCV antigens are not expressed without administration of an adenovirus that expresses Cre DNA recombinase. This transgenic mouse line showed overt immune responses that were correlated with the status of hepatitis. This model might be useful for understand-

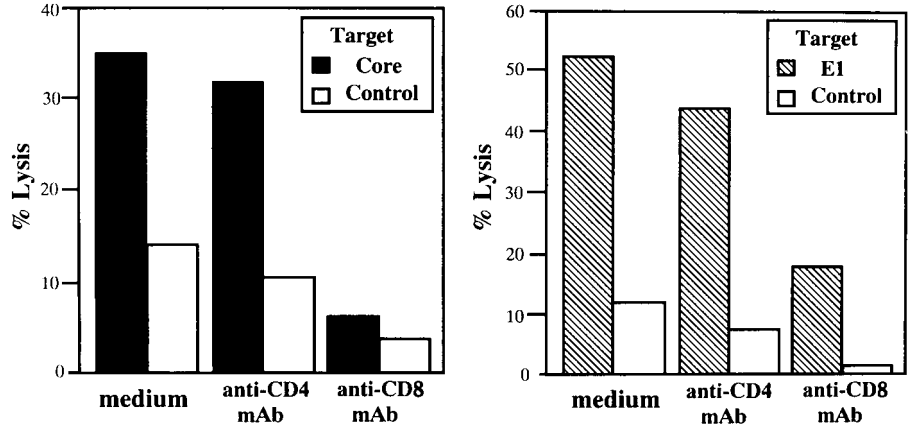

FIG. 4. HCV-specific lysis by intrahepatic pEFCE1E2 inoculation is mediated by $CD8^+$ cells. Spleen cells from mice intrahepatically inoculated with pEFCE1E2 were stimulated with CTL epitope peptide, and lytic activities against peptide-labeled target cells were assessed in the presence of anti-CD4 mAbs or anti-CD8 mAbs. The effector:target ratio was 80:1. The data represent the mean percentage of the specific lysis values obtained from five mice.

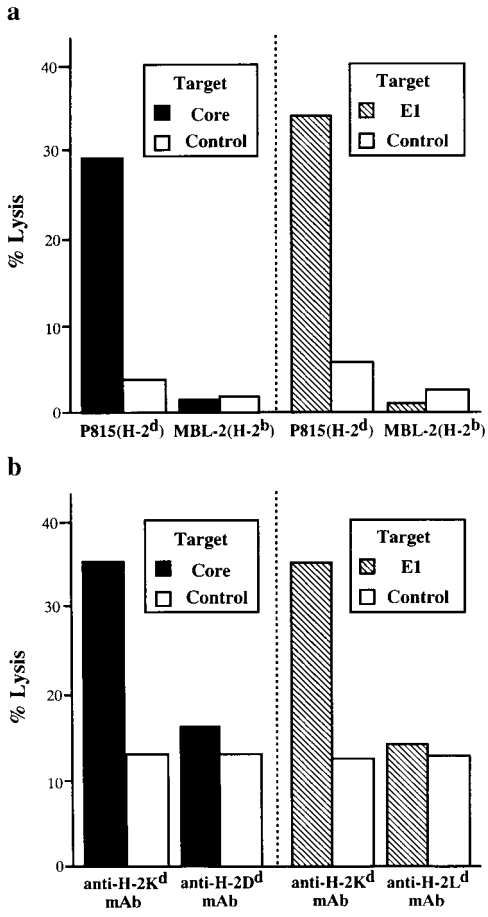

FIG. 5. HCV-specific lysis was restricted by MHC class I. (a) HCV-core or HCV-E1 CTL epitope peptides stimulated spleen cells from mice intrahepatically immunized with pEFCE1E2 lysed HCV-core or HCV-E1 CTL epitope peptide-pulsed H-2^d but not H-2^b target cells. Effector cells were examined for their lytic activities against HCV-core or HCV-E1 CTL epitope peptide-pulsed H-2^b or H-2^d target cells. (b) HCV-core or HCV-E1 CTL epitope-specific lytic activities were inhibited by anti-H-$2D^d$ or anti-H-$2L^d$ mAb, respectively. Effector cells were assessed for HCV-core or HCV-E1 CTL epitope-specific lytic activities in the presence of anti-H-$2K^d$, anti-H-$2D^d$, or anti-H-$2L^d$ mAb. Closed and hatched bars indicate the lysis of target cells labeled with HCV-core or HCV-E1 CTL epitope peptide, and open bars indicate the lysis of control peptide-labeled target cells. The effector:target ratio was 80:1. The data represent the mean percentage of the specific lysis values obtained from five mice.

ing the correlation between immune responses and hepatitis if there is no influence of adenovirus infection in the model.

Direct gene transfer into liver cells by *in vivo* electroporation has been reported to be a useful method for expressing antigens (Suzuki *et al.,* 1998). In the present study, we showed the expression of HCV antigens in mice intrahepatically inoculated with plasmid having the HCV structural protein gene. There were some differences in the pathological features of the livers of these mice from those of HCV-infected humans. Our model showed the expression of HCV antigens not only in hepatocytes but also in other cells (e.g., sinus endothelial cells), unlike that in the liver of HCV-infected humans. Another difference between our experimental mice and HCV-infected humans was the extent of hepatitis. Since electrotransfection efficiency was observed in only a small area surrounding the electrode needles, mice intrahepatically inoculated with HCV genes did not show any increment in the serum levels of parameters affected by hepatitis, such as alanine aminotransferase (data not shown). These characteristics were dependent on the administration of DNA by using the *in vivo* electroporation method, and it is thought that there is still room for improvement in this technique.

Cellular immune responses, particularly those mediated by $CD8^+$ CTL, may generally play important roles in the pathogenesis and control of HCV infection (Nelson *et al.,* 1997). For the purpose of understanding these roles, animal models should elicit HCV-specific CTL responses; however, most of the transgenic mouse lines thus far established have not clearly shown the existence of HCV-specific CTL. Moreover, the chimpanzee, which has been used for studies on the replication of and the immune response against HCV, is difficult to employ for laboratory experiments. The present study demonstrated the elicitation of HCV-specific $CD8^+$, MHC class I-restricted CTL in mice intrahepatically inoculated with HCV DNA. The contribution of such CTL to the pathogenesis of HCV infection can now be assessed in an intrahepatically immunized mouse model.

MATERIALS AND METHODS

Plasmid

The HCV genotype 1b cDNA was isolated from a Japanese individual with chronic hepatitis C by the method previously described (Wakita and Wands, 1994). The cDNA was ligated into pEF321-Neo (Kim *et al.,* 1993). The plasmid pEFCE1E2 encoding structural proteins, core protein, and E1 and E2 proteins (amino acids 1–705) of type 1b HCV under the control of the EF1-α promoter (Nelson *et al.,* 1997) was used for immunization (Fig. 6). Plasmid DNA was amplified in *Escherichia coli* and was purified using a Qiagen purification kit (Qiagen, Inc., CA). It has been reported that a single intramuscular immunization with pEFCE1E2 elicits HCV-specific CTL (Nishimura *et al.,* 1999). An empty plasmid, pEF321-Neo, was used as a control.

Immunization

Six- to eight-week-old BALB/c mice were given a single intramuscular or intrahepatic injection of the plasmids (10 μg/mouse), and then an electric pulse was immediately applied by an Electric Square Porator (T820; BTX, San Diego, CA). Pulses were delivered to the liver or

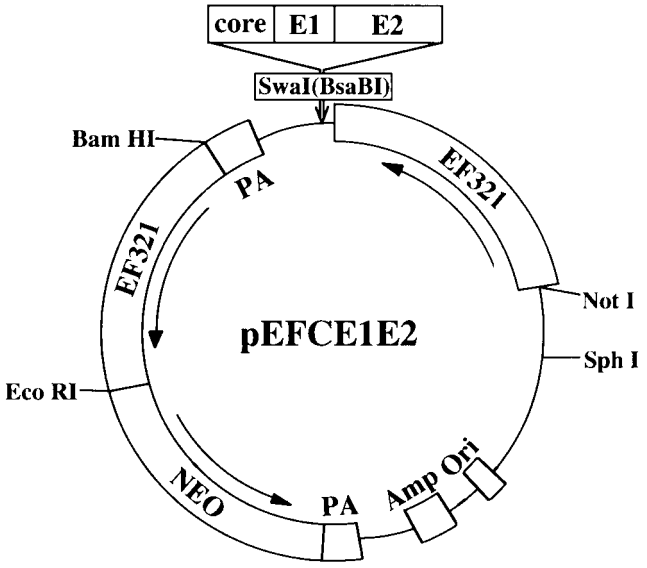

FIG. 6. Scheme of the HCV structural protein expression plasmid pEFCE1E2. HCV-core, -E1, and -E2 regions were inserted under the EF-1α promoter.

muscle using a pair of electrode needles. Eight electric pulses were administered at a rate of one pulse per second. Pulses were delivered to the left lobe of the mouse liver or to the gastrocnemius muscle, which had been injected between electrode needles separated by a distance of approximately 5 mm. Electric pulses were delivered twice to the left lobe of liver. In the second administration, the axis of the electrode needles was changed to an angle of 90° to the axis of electrode needles in the first electric administration. The electric pulse was 99 ms in duration and the voltage was 50 V. The resistance was monitored with a graphic pulse analyzer (Optimizer 500, BTX).

Tissue preparation

Tissue samples from the liver were fixed in 4% phosphate-buffered paraformaldehyde solution, embedded in paraffin wax, and cut into serial sections of 4 μm in thickness for histopathologic and immunohistochemical examinations. The serial sections for histopathologic examination were stained with hematoxylin and eosin (HE).

Immunohistochemical analysis

After deparaffinization, sections were microwaved for 10 min in sodium citrate buffer (0.01 M, pH 6.0). The sections were incubated in 3% H_2O_2 methanol for 30 min to quench endogenous peroxidase activity and then washed three times for 5 min in 0.05 mol Tris–HCl (pH 7.6, with 0.15 M NaCl). The sections were then incubated with Block Ace (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) at 37°C in a moisture chamber for 30 min to prevent nonspecific reactions. Subsequently, the sections were incubated with the following mAbs (diluted 1:100) at 37°C overnight: C515 (anti-HCV-core; Anogen, Ontario, Canada), E1-299 (anti-HCV-E1; Austral Biologicals, CA), and E2-917 (anti-HCV-E2; Austral Biologicals). After being washed the sections were incubated with a secondary antibody (diluted 1:1000) (rabbit anti-mouse IgG; ICN Pharmaceuticals, Irvine, CA) and treated with 2% avidin–botin complex. They were then reacted with 0.05% 3,3′-diaminobenzidine tetrachloride to visualize the bound antibodies.

Generation of CTL effector cells

Effector cells were derived from spleen cells as precursor CTL. Aliquots of 5×10^6 spleen cells were cocultured with 2.5×10^6 mitomycin C-treated autologous spleen cells labeled with a peptide (20 μg/ml) or infected with a rVV expressing HCV E2 (m.o.i. 5) at 37°C in a CO_2 incubator. The effector cells generated were harvested after 5 days of culture.

Cytotoxicity assay

The target cells were MHC-matched (P815) or -mismatched (MBL-2 and FBL-3) tumor cells incubated at 37°C under a 5% CO_2 atmosphere with the HCV-core CTL epitope (HCV core 133–142, LMGYIPLVGA) (Saito *et al.,* 1990), HCV-E1 CTL epitope (HCV E1 315–322, GHRMAWDM) (Bruna-Romero *et al.,* 1997), control peptide (human immunodeficiency virus residues 308–322; RIQRGPGRAFVTIGK) at 10 μg/ml, or rVV expressing HCV E2 or an irrelevant virus gene (2×10^7 PFU) for 16 h. Then the target cells were washed and labeled with ^{51}Cr. The ^{51}Cr-labeled target cells were incubated for 5 h with effector cells. Spontaneous release varied from 5 to 15%. Percentage lysis was calculated as [(experimental release − spontaneous release)/(100% release − spontaneous release)] × 100. All experiments were performed more than three times, and each group consisted of five mice.

Blocking of cytolysis

Blocking of cytolysis was performed according to the method reported previously (Van Snick *et al.,* 1982). Effector cells were preincubated with anti-CD4 mAb (GK1.5) or anti-CD8 mAb (Lyt2.2) at a 1:50 dilution at 4°C for 1 h, and then the labeled target cells were added. Blocking of cytolytic activities by these mAbs was assessed by a 5-h ^{51}Cr release assay.

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